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**14. ABSTRACT** Estrogens and antiestrogens are important in the development, treatment and possible chemoprevention of breast cancer. Many reports have suggested that there are a number of signaling responses to estrogens and antiestrogens that are independent of the classic transcriptional model of estrogen receptor action, but there is much controversy as to the mechanisms underlying these responses and their overall relevance to breast cancer. This proposal aims to design and use selective chemical probes to answer those questions. A screening panel has been synthesized to probe the role of these estrogen responses in breast cancer proliferation and resistance chemotherapy. We have learned more about the structure activity relationships necessary for potent ER modulators. We have discovered new responses via serum response factor that appear to be downstream of rapid estrogen signaling. We have discovered that tamoxifen-polymer conjugates are efficiently taken up by breast cancer cells in a specific manner and possess highly potent biological activity.
We have also generated the first tamoxifen-conjugated nanoparticles and have demonstrated their biological activity. Ongoing studies will use these compounds and constructs to better understand estrogen signaling and make better therapeutic and chemopreventive agents for breast cancer.

**15. SUBJECT TERMS**
estrogen, estrogen receptor, nongenomic signaling, SERMs, tamoxifen, raloxifene, synthesis, drug discovery, serum response factor

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**Introduction**

Estrogens and antiestrogens are of utmost importance in the development, treatment and possible chemoprevention of breast cancer. Although much progress has been made in understanding the mechanisms by which estrogen and SERMs function, a class of mechanisms that is getting increased scrutiny is the so-called “non-genomic” response that is due to modulation of cell signaling pathways other than direct transcriptional regulation. There is much debate as to the receptors responsible for these responses and the mechanisms by which they operate. This proposal aims to design and use selective chemical probes to begin to answer those questions. In particular, estrogen responses related to breast cell proliferation and resistance to apoptosis will be studied using a variety of chemical probes including polymer-based drugs designed to test the potential role of cell surface estrogen receptors.

**Body**

This project has focused on developing and testing chemical probes of rapid responses to estrogen relevant to the treatment and chemoprevention of breast cancer. The proposed project was broken into 4 main tasks:

1. Determine the effects of the ligand structure on both rapid signaling and estrogen receptor-mediated transcription by testing a screening library of various known and novel estrogen response modulators in a number of assays.
2. Test the potential role of estrogen receptor alpha (ER$\alpha$) or ER$\beta$ in rapid signaling, by performing assays with various N-terminal deletion and chimera mutants of ER$\alpha$ and ER$\beta$.
3. Test the role of cell surface receptors in rapid estrogen signaling, by developing cell-impermeable, non-proteinaceous estradiol conjugates.
4. Test the potential role of rapid estrogen signaling in breast cancer proliferation and survival, by treating various breast cell lines with selective compounds discovered above and measuring changes in cell growth, cytotoxicity and apoptosis

As will be described in the rest of this report, much significant progress has been made with task 1 and 3 while only some progress in the other tasks has been made due to some difficulties encountered in assay development and in some of the findings in the development of the compounds for task 3. Solutions have since been found to circumvent those difficulties and it is expected that tasks 2 and 4 can now be completed as described. As will be described later in the report, it is clear from our second year of work on this project that it is very difficult to purely separate these “rapid” responses from other downstream effects of receptor action and that the most appropriate approach is one that investigates the integration of rapid responses arising from receptor crosstalk with other downstream events. This has important implications in ligand design of hormone-based breast cancer treatments. An update on the research follows and is organized by the specific tasks of the statement of work.

*Task 1. Determine the effects of the ligand structure on both rapid signaling and*

*estrogen receptor-mediated transcription by testing a screening library of various known and novel estrogen response modulators in a number of assays.*

*<u>Task 1a.</u> Generate the screening library by synthesizing a small number of estradiol and triphenylethylethylene analogs and combining it with commercially available and previously synthesized compounds. (Months 1-6)*
The initial screening proposed is shown in **Figure 1** including a few compounds that were not in the initial panel that have since been synthesized.

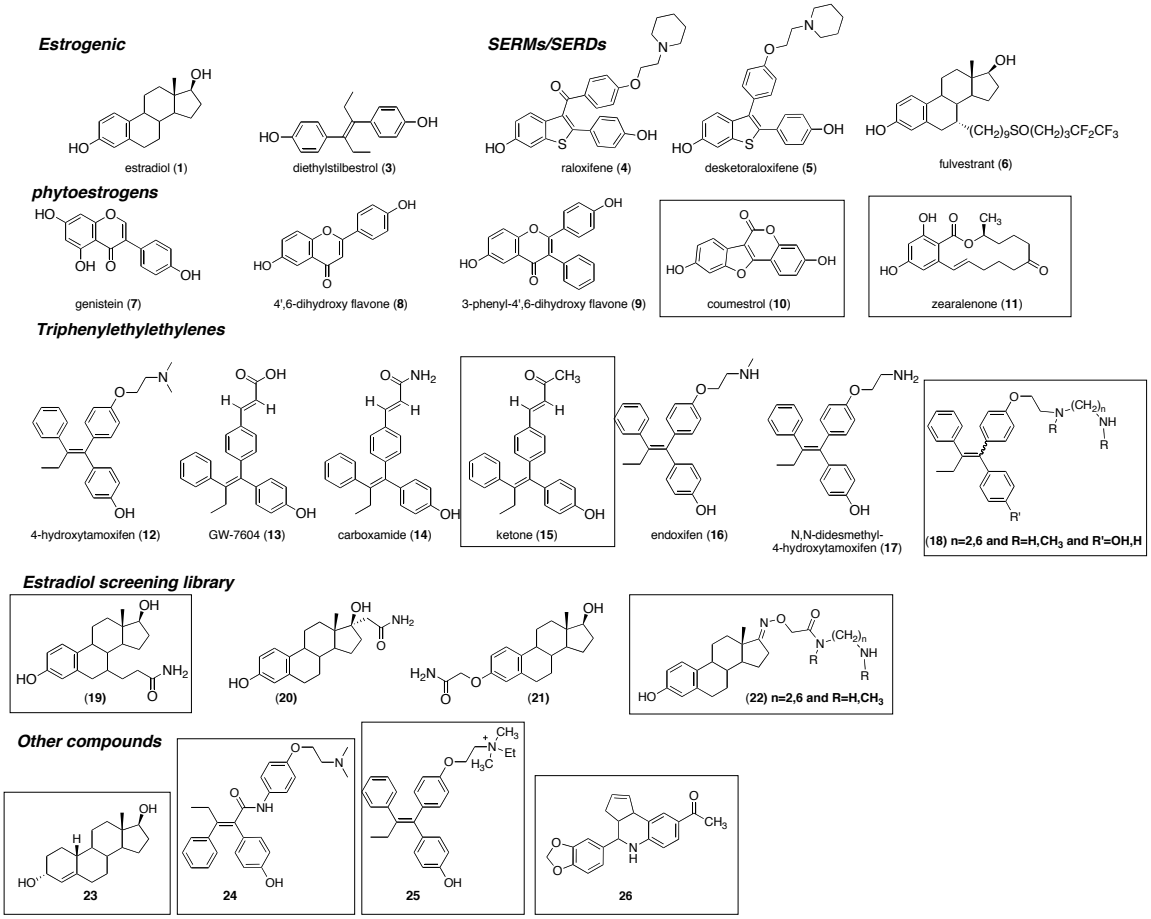
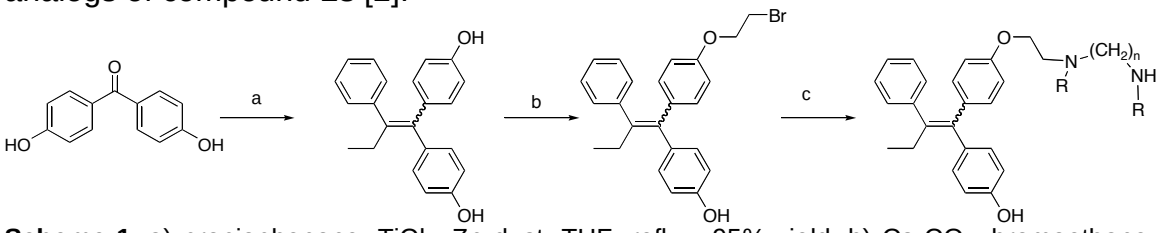



**Figure 1**. Initial screening panel. Compounds that have been synthesized that were not part of the proposed panel are boxed in solid boxes.

In generating this panel of compounds, a number of new synthetic approaches were developed. Below is a description of these new discoveries.

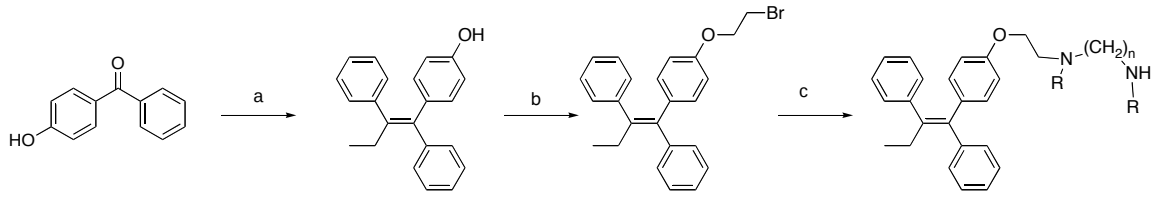
*<u>Synthesis of triphenylethylethylenes</u>*
In order to generate new side chain analogs of 4-hydroxytamoxifen, a new synthesis was developed that greatly simplified the approach compared to previous syntheses. The first approach was developed using a monoalkylation followed by McMurray coupling to generate analogs with different side chain moieties to make compounds **16**,**17** and **18** (R=H, R'=OH, n=2). Another synthesis was developed to overcome inadequacies of the original plan by modifying a previously reported synthesis of 4-hydroxytamoxifen (**Scheme 1**).

[1]. This procedure allows for the facile generation of gram quantities of 4-hydroxytamoxifen analogs and has been used to make a number of different analogs of compound **18** [2].




**Scheme 1.** a) propiophenone, $TiCl_4$, Zn dust, THF, reflux, 95% yield; b) $Cs_2CO_3$, bromoethane, DMF, reflux, 70% yield; c) $RNH(CH_2)_nNHR$, THF, 80˚, sealed tube, 100 % yield

The approach has also been modified (**Scheme 2**) to synthesize analogs based on tamoxifen in addition to 4-hydroxytamoxifen (compound **18**, R=H or $CH_3$, R'=H, n=2 or 6). Whereas the synthesis of 4-hydroxytamoxifen analogs always resulted in a mixture of double bond stereoisomers that rapidly interconvert at room temperature, the McMurray coupling to form tamoxifen analogs gave a 1:1 mixture of E and Z stereoisomers that do not interconvert. The desired Z isomer could be selectively recrystallized from isopropanol.




**Scheme 2.** a) propiophenone, $TiCl_4$, Zn dust, THF, reflux, 45% yield of Z isomer; b) $Cs_2CO_3$, bromoethane, DMF, reflux, 95% yield; c) $RNH(CH_2)_nNHR$, THF, 80˚, sealed tube, 100 % yield

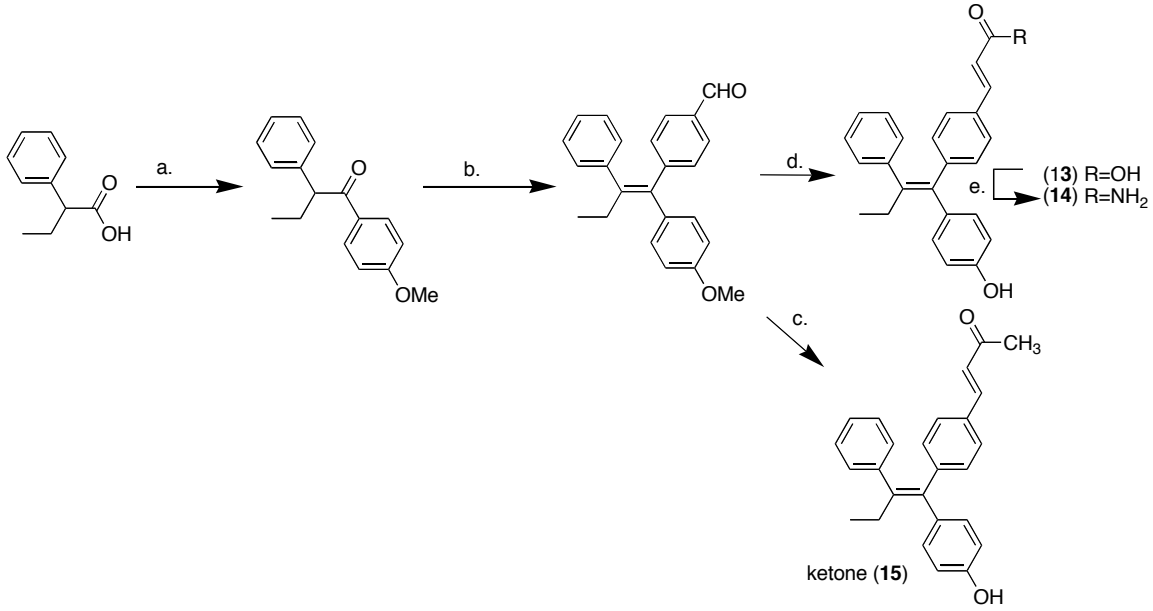
*New synthesis of GW-7604 analogs*

Another set of compounds in the library based on the triphenylethylethylene scaffold is the GW-7604 series (**13**,**14** and **15**). These compounds have been synthesized using a previously reported procedure, but an improved synthesis is needed [3]. These compounds are interesting due to recent reports that their ER-modulating properties are more like pure antiestrogens like fulvestrant than SERMs like tamoxifen [4]. A new synthesis was designed and executed (**Scheme 3**). This synthesis also allowed for more facile introduction of different side chains for this class of compounds.

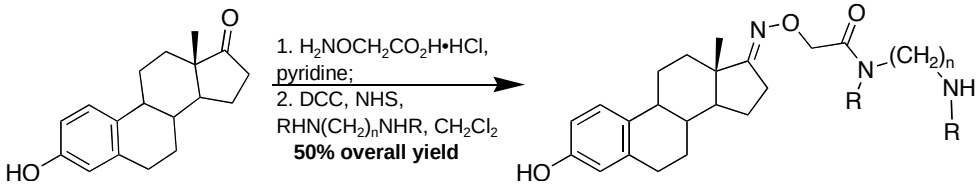
*Estradiol screening panel synthesis*

The initial plan was to attach acetamide groups to 5 different positions on the estradiol steroid scaffold- 3, 6, 7, 11, and 17. Synthesis of the 3-substituted analog was straightforward after modifying a previously reported procedure [5], but as will be described later, generated a compound that was unable to bind to estrogen receptor alpha . As a result, that substitution point is not being pursued at the current time.

**Scheme 3.** (a). 2-phenylbutyric acid, trifluoroacetic acid anhydride, phosphoric acid, anisole, 10 ˚C, 100% yield [6] (b). (i)., THF, magnesium, 4-bromobenzaldehyde diethyl acetal; $H_3O^+$(ii). HCl, ethanol, reflux, 76% yield. (c). (i). diethyl (2-oxopropyl)phosphonate, potassium bis(trimethylsilyl)amide, THF, -78 ˚C to room temp. (ii). $BBr_3$, $CH_2Cl_2$, 0 ˚C, 54% yield. (d). (i).trimethlyphosphonoacetate, potassium bis(trimethylsilyl)amide, THF, -78 ˚C to room temp. (ii). KOH, EtOH/THF, reflux (iii). $BBr_3$, $CH_2Cl_2$, 0 ˚C, 37% yield. (e). EDC, HOBT, $Et_3N$, $NH_4OH$, DMF, 80% yield




Substitution at 17 has been accomplished through two different routes. The first involved Grignard alkylation of estrone to generate a 17-$\alpha$ alkyl group. This compound only has moderate affinity for the receptor. As a result, another 17-substituted compound was made by forming the oxime at the 17-position starting from estrone (**Scheme 4**). Modifying a previously reported procedure [7], a number of analogs have been synthesized containing this substitution and they have been found to possess high affinity for the estrogen receptor.

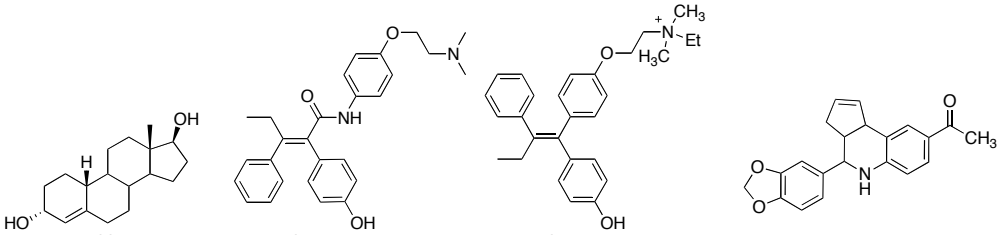



**Scheme 4.** In this case, compounds have been made with R=H or $CH_3$ and with n=2 or 6.

Difficulties were encountered with synthesizing analogs derivatized at the 6, 7 or 11 position. The decision was made to focus on substitution at the 7 position because these analogs look most like fulvestrant (also known as ICI 182,780), an antagonist in many rapid response assays. A new synthetic route based on the most recent literature report is being started and there is hope that the problems can be solved [8]. Once this 7-substituted analog is complete, the screening panel will be entirely finished.

*New compounds*

Since the submission of this proposal, there have been reports of compounds with no reported activity in regulating estrogen receptor-mediated transcription, but still possessing the ability to stimulate rapid signaling. The first molecule in this class was the estren derivative, 4-estren-3α,17β-diol (**23**), which was shown

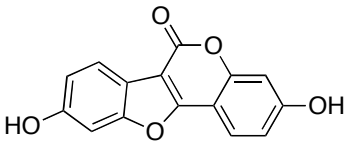



**23** **24** **25** **26**

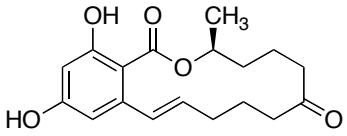
to selectively activate rapid signaling in bone without much transcriptional modulating activity [9]. The utility of this compound in rapid responses in other tissues has yet to be explored. The other compound reported to be in this class is STX (**24**), a compound with the opposite alkene stereochemistry as tamoxifen. This compound mimicked estradiol's ability to rapidly reduce the potency of the $GABA_B$ receptor agonist baclofen to activate G-protein-coupled, inwardly rectifying K+ channels in hypothalamic neurons, a model of estrogen-induced prevention of hot flashes [10]. This compound has not been tested in any other models of integrated estrogen signaling and it could also be possible that it acts through a receptor other than the nuclear receptor– STX has no discernable affinity for the estrogen receptor alpha or beta in vitro. Another compound, known as G-1 (**25**), has been shown to selectively activate GPR30, an orphan GPCR that has been shown to be activated by estrogens and might be responsible for some nongenomic effects [11-13]. All of these compounds have been either purchased or synthesized and will be used in later experiments.

In addition, some people have focused on making analogs of known ligands of the estrogen receptor and somehow restricting their access across the plasma membrane. Every compound with transcriptional activity has good cell permeability by definition because the receptor is intracellular, but modifying the compound so that it is charged can restrict diffusion across the membrane. Q-Tam (**26**), a quaternary ammonium salt of tamoxifen, was found to induce apoptosis in damaged mammary epithelial cells through direct decrease in Akt phosphorylation [14]. This compound has been synthesized and will be used in later experiments.

Finally, a number of phytoestrogens have also been proposed to possibly modulate breast tumor proliferation [15]. A number of these compounds based on flavinoid structures were already included in the screening panel, two more non-flavinoid compounds were added to the panel, coumestrol (**10**) and the mycotoxin zearalenone (**11**). These compounds have been purchased and will be used in later experiments.




coumestrol (**10**)




zearalenone (**11**)

*<u>Task 1b</u> Test the ability of the compounds to modulate nuclear-initiated signaling by performing reporter gene assays at classic ERE promoters or nonclassical AP-1 promoters.*

The overall goal of this proposal is to develop chemical tools to study rapid responses to steroid hormones. Key to accomplishing this goal is being able to correlate the ability of the compounds to bind to the nuclear receptor in vitro with the ability to directly activate the kinases and regulate the gene transcription by different transcription factors in cells. Therefore, assays for all three activities need to be developed and will be described below.

*<u>Nuclear receptor binding</u>*

There have been many assays reported to measure the binding of compounds to either the estrogen receptor. Most involve competition experiments using purified receptor or crude cell extracts and radiolabeled steroid hormone. We have used a commercially available assay kit based on fluorescence polarization with purified recombinant estrogen receptor alpha and beta and a fluorescent hormone analog. The assays were performed in 96 well plates and are fairly routine. A standard competition curve for estradiol is shown in **Figure 2**. **Table 1** lists the binding affinities of any compound that has not been reported previously in the literature. From the data, it is clear that the original plans for sites were conjugation were not going to result in compounds with enough affinity, so new conjugates were synthesized quickly and high affinity compounds were produced. It also appears that the length of the linker arm extending away from the compound is not crucial in obtaining high affinity compounds.

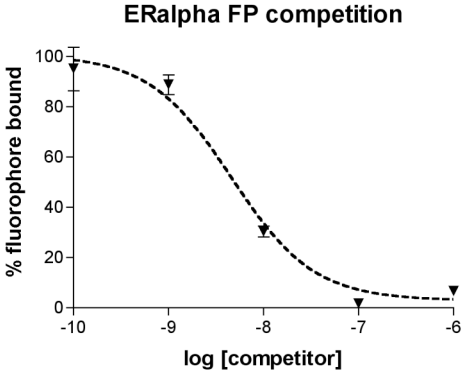



**Figure 2**. Estrogen receptor alpha competition binding experiment vs. 2 nM Fluormone™ with estradiol as the competing ligand. Each point represents three separate samples

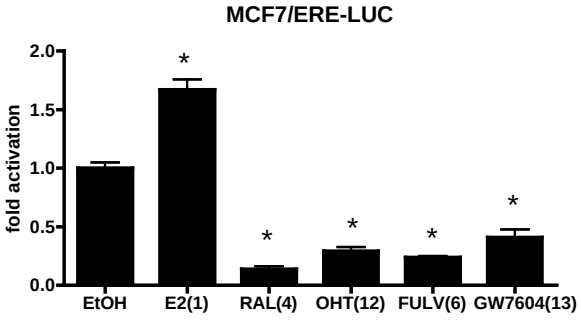


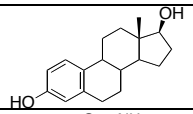
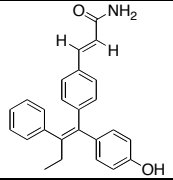
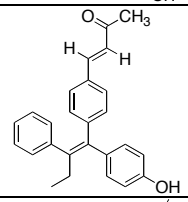
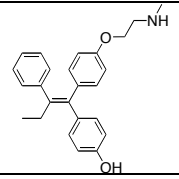

**Figure 3**. Luciferase reporter gene assay using vitellogenin-ERE promoter in transiently transfected MCF-7 cells. The number next to each compound refers to the structures in Figure 1.

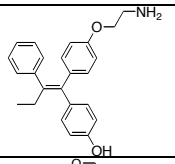
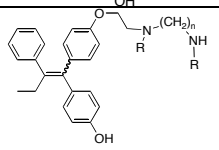
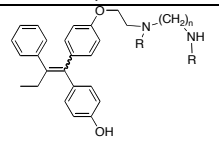
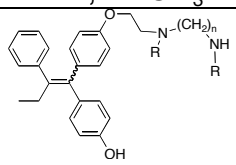
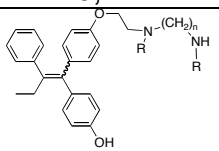
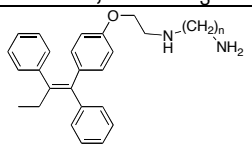
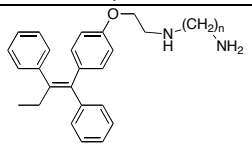
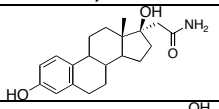
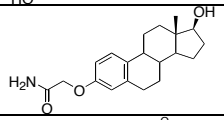
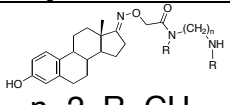
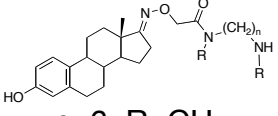
*Luciferase reporter gene assays*

A key component of this project is measuring the estrogen receptor-mediated transcriptional activity of the compounds. For estrogen receptor, MCF-7 cells, which contain both ERα and ERβ, were transiently transfected with a luciferase reporter plasmid controlled by a simple estrogen response element (ERE)-containing promoter from the upstream region of the vitellogenin gene. The ER-negative HeLa cell line was also used for these experiments, but an expression plasmid for either ERα or ERβ was cotransfected with the reporter plasmid. A dual luciferase reporter gene system was used to normalize for transfection efficiency, meaning that an enzymatically orthogonal form of luciferase from a different species was cotransfected on a constitutively active expression plasmid. The DNA was transfected into the cells using Lipofectamine 2000 and standard protocols. After transfection, the cells were treated with drug for 1-2 days and the activities of the two luciferases were measured independently using a commercial kit. This assay is quite robust and reliable.

**Figure 3** and **Table 1** show the fold activation of transcriptional activation at the ERE response element of a number of the compounds from the screening panel. As is to be expected, most of the SERMs and antiestrogens act as antagonists and estradiol and genistein act as agonists. The only real surprise was the activity of estren. This compound was reported to have no activity with estrogen receptor, but it is clear that there is some agonist activity. The reason behind this activation is still being explored. The activity of antagonists can be also be measured by performing a competition experiment with 10 nM estradiol. **Table 1** lists the inhibitory potencies of any compound that has not been reported previously in the literature. From the data, the potency of the compounds at repressing ER-mediated transcription correlates with binding affinity.

**Table 1.**

| compound | | Ki (nM) | IC50 (nM) |
|---|---|---|---|
| estradiol **(1)** |  | 6.3 ± 0.2 | N.D. |
| **14** |  | 35 ± 17 | 110 ± 20 |
| **15** |  | 25 ± 12 | 55 ± 10 |
| **16** |  | 8.5 ± 3.9 | 40 ± 10 |

| | | | |
|---|---|---|---|
| **17** |  | 48 ±5 | 800 ± 400 |
| **18** |  n=2, R=H | 32 ± 10 | 150 ± 50 |
| **18** |  n=2, R=$CH_3$ | 3.4 ± 2.1 | 39 ± 12 |
| **18** |  n=6, R=H | 9.8 ± 6.2 | 85 ± 55 |
| **18** |  n=6, R=$CH_3$ | 6.2 ± 4.6 | 126 ± 33 |
| **18** |  n=2, R=H | Yet to be det'd | 150 ± 24 |
| **18** |  n=6, R=H | Yet to be det'd | 110 ± 32 |
| **20** |  | 850 ± 75 | 3275 ± 200 |
| **21** |  | 1100 ± 100 | > 10 $\mu$M |
| **22** |  n=2, R=$CH_3$ | 9 ± 4 | 13 ± 6 (weak agonist) |
| **22** |  n=6, R=$CH_3$ | 22 ± 8 | 32 ± 11 |

*Task 1c. Test the ability of the compounds to mimic estrogen's ability to inhibit apoptosis in breast cancer by treating a breast cell line with the compounds in the presence of taxol and testing for both early and late apoptosis events.*

One of the key aspects of this project is determining the effect of various compounds on the tolerance to apoptosis that estradiol confers to ER positive breast cancer cells. We started these assays early in this project but have had some difficulty in obtaining reproducible results. Early efforts focused on using a previously reported assay for caspase 9 activity to indicate early events in apoptosis [16]. This assay has not been successful in our laboratories. We have also performed fluorescence microscopy studies to look at annexin V binding to the cell surface- a marker for the late stages of apoptosis. While some data were generated with this approach, a flow cytometry based approach will be much more statistically significant and a student is currently undergoing training in the Purdue Cytomics facility to execute these experiments.

*Task 1d. Test the ability of the compounds to mimic estrogen's ability to rapidly initiate kinase signaling cascades known to be important in cell proliferation by treating different cell lines with the compounds and testing for modulation of kinase pathways starting with MAP kinase.*

While there are many different assays that can be run to measure direct activation of the three kinases, the goal of this proposal is to start with assays that are well established. For our initial studies, we have decided to focus on the direct activation of ERK1/2 in two cell lines– the ER-positive breast cancer cell line MCF-7 and the ER-negative cell line MDA-MB-231 with or without transfected ER$\alpha$. For measuring direct activation of ERK1/2, the cells are serum-starved for 3 days to quiet any background MAPK signaling. Cells are then treated with drug for various time points, then the cells are lysed and the cell lysates are analyzed for total ERK and phosphorylated ERK (pERK) using previously reported Western blotting procedures [17]. While some stimulation with different compounds was seen in transfected MDA-MB-231 cells, it was clear from later experiments with fluorescent proteins that the transfection efficiency of this model system is relatively poor (less that 30%) and that most of the MDA-MB-231 cells were untransfected. As a result, experiments with transfected MDA-MB-231 cells were abandoned. In contrast, in ER-positive MCF-7 cells, estradiol stimulated ERK phosphorylation about as strongly as epidermal growth factor (EGF), which is consistent with previous reports. [18] Care is taken to not exceed an ethanol or DMSO concentration in the media over 0.01% since higher levels of either solvent can stimulate ERK phosphorylation. The specificity of the MAPK pathway for ERK phosphorylation is shown by the inhibition of estradiol stimulation by the MEK inhibitor PD98059. Specificity for an estrogen response is shown with the inhibition of estradiol stimulation using the antiestrogen fulvestrant. The time course of activation was also determined in MCF-7 cells and is shown in **Figure 4**. The ERK activation after dosing with

estradiol was maximal at 5-10 minutes with most of the activation returning back to baseline after 15 minutes.

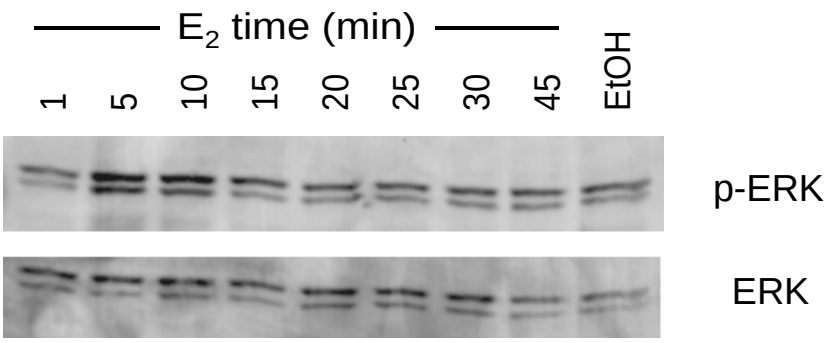



**Figure 4.** ERK phosphorylation in MCF-7 cells after doing with 10 nM estradiol.

The effects on ERK activation of a number of other compounds in the screening library are shown in **Figure 5**. Tamoxifen, 4-hydroxytamoxifen, estren, raloxifene and desketoraloxifene all elicited ERK phosphorylation after 15 minutes in MCF-7 cells. This activation was MAPK specific as it was inhibited by PD98059. All of the responses were estrogen receptor specific in that they activation could be blocked by fulvestrant (also known as ICI 182,780) except for the tamoxifen compounds. It appears that ERK activity *increases* in the presence of fulvestrant. This experiment has been repeated and the same result is obtained. Work is currently underway to try to understand the origin of this effect with a focus on previously reported antiestrogen binding site on cells [19].

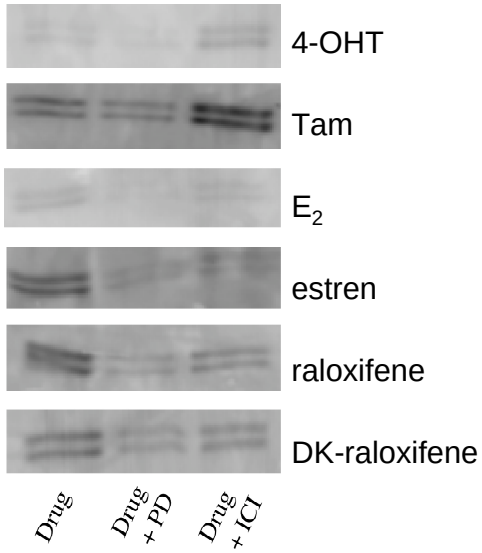



**Figure 5.** phospho-ERK levels in MCF-7 cells after 15 minute exposure to different estrogen receptor ligands. **column 1**-100 nM compound; **column 2**- compound + PD 98059; **column 3**-compound + fulvestrant (ICI 182,780)

*Problems with activation assays and potential solutions*

The major obstacle facing this project currently is the lack of consistent and vigorous activation of the MAPK pathway. The fold activation is usually 2-3 fold over baseline, but many times the baseline seems to be much higher than

normal and no ER-induced activation is seen. Various types of serum starved and serum-free conditions have been tried as well as cell lines expressing high levels of Her2/neu. We have tried other antibodies as well as immunoprecipitating ERK and performing kinase enzymatic assays. The same issue has arisen looking at the phosphorylation of Akt, reported to be another downstream effector of nongenomic estrogen signaling. Thus far, we have not found a technique for either signaling pathways that gives highly reproducible results.

There are a number of other possible solutions in the literature that we have pursued. One involved making a form of the estrogen receptor that localizes to the membrane. This receptor lacked the nuclear localization site and included additional myristoylation and prenylation sites and was reported to have strong ERK activation properties [20]. In order to confirm that the receptor localized to the membrane, we first ran a luciferase reporter gene experiment with the reporter gene coupled to a classic estrogen response element-controlled promoter. In reported work by others, this receptor did not regulate transcription at an ERE promoter [20]. In our work, estradiol was still able to activate transcription from the ERE promoter, suggesting that there was perhaps still some nuclear activity. We then constructed a version of the membrane-localized receptor fused to green fluorescent protein (GFP) and transfected cells with this expression plasmid. While fluorescence was observed at the membrane, significant fluorescence was also observed in the nucleus both before and after addition of estradiol, suggesting that the targeting strategy was unsuccessful. As a result, this approach was abandoned.

*Serum Response Factor Modulation*

We also tried luciferase reporter gene assays using a number of different downstream transcription factors that were reported to be sensitive to changes in MAPK or PI3K activation. We focused our attention on the transcription factors that regulate transcription at the serum response element (SRE), Elk-1 and the serum response factor (SRF). Genes under control of SRE containing promoters have been reported to be expressed very quickly after estradiol treatment and do not appear to involve direct estrogen receptor modulation of the promoter. Rather, Elk-1 and SRF are modulated by estrogen receptor through the MAPK and PI3K pathways (**Figure 6**) [21,22]. In order to determine whether our screening panel had unique modulatory properties through these pathways, an SRE luciferase reporter plasmid, an SRF reporter plasmid and Elk-1 reporter plasmids (the Elk-1 reporter system consists of two plasmids) were transfected into ER-positive MCF7 cells, and the ER negative cell lines MDA-MB-231 and SKBR3. For the ER-negative cell lines, expression plasmids for either ER$\alpha$ or ER$\beta$ were cotransfected. The SKBR3 cell line was chosen because it is known to have high levels of growth factor receptor and might be expected to have elevated MAPK and PI3K signaling.

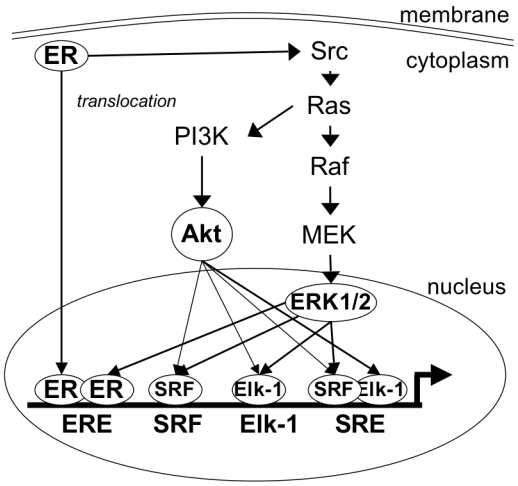



**Figure 6.** Proposed regulation of the transcription factors SRF and Elk-1 by crosstalk of estrogen receptor with the MAPK and PI3K pathways. SRF and Elk-1 work together at the serum response element (SRE) when they are expressed in the same cell.

The Elk-1 reporter plasmids did not show significant ligand-dependent modulation in any of the cell lines tested. In contrast, the SRF-controlled reporter plasmids did show significant ligand responses in an ER-dependent manner that was also dependent on cell context and this activity was also seen with the SRE reporter, although the relative SRF reporter response compared to vehicle was greater than the relative SRE response compared to vehicle. Using a dual luciferase reporter assay to normalize for transfection efficiency, estradiol was found to increase SRF-mediated transcription (**Figure 7**), consistent with a previous report that indicated that SRF could be modulated by estrogen receptor through crosstalk with MAPK and PI3K [22]. Testing the other compounds in the panel, however, revealed that the selective estrogen receptor modulator (SERM) raloxifene repressed the basal activity of SRF. Somewhat surprisingly, other compounds such as the SERM 4-hydroxytamoxifen and the selective estrogen receptor downregulators (SERDs) fulvestrant and GW-7604 did not have significant effect on basal SRF activity.

A recent study has suggested that the orphan G-protein coupled receptor GPR30 regulates the transcription of *c-fos*, possibly by modifying the activity of SRF and Elk1 at the SRE contained in the c-fos promoter region [23]. In order to test the possible involvement of GPR30 in mediating the effects of estradiol and raloxifene on SRF activity, the reporter plasmids were tested in the ER-negative, GPR30-positive SKBR3 and MDA-MB-231 breast cell lines. To confirm that no endogenous ER activity was present in the either cell line, an ERE-containing reporter plasmid was first transfected into the cells and the cells were treated with either estradiol or raloxifene. No significant ligand-dependent response was seen in either cell line unless an expression plasmid for ER$\alpha$ was cotransfected (**Figure 8A and B**). Convinced that no appreciable amount of ERα or ERβ activity was present, we transfected the cells with the SRF reporter plasmid and treated the cells with estradiol or raloxifene. As was seen with the ERE reporter plasmid, no significant ligand-dependent response was seen (**Figure 8C and D**),

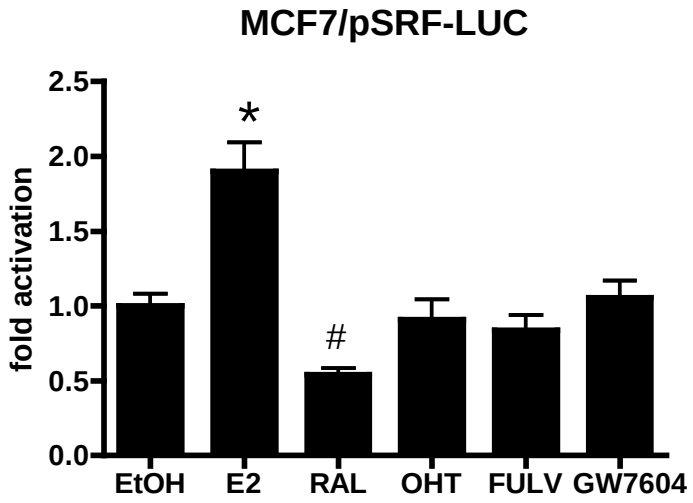



**Figure 7.** Effects of some of the screening panel on MCF-7 cells transfected with a luciferase reporter plasmid containing an SRF binding motif. The cells were treated with drug for 24 hours in charcoal-stripped media. The results were obtained using a dual luciferase kit to normalize for cell number and transfection efficiency and the results are displayed as fold activation over the ethanol vehicle. All drugs were tested at 1 μM concentration. Activity is reported as fold activation compared to the ethanol vehicle. * represents responses differing from the vehicle response with $p < 0.01$. # represents responses deviating from the vehicle response with $p < 0.05$.

suggesting GRP30 does not regulate SRF activity in these cells. When the cells were cotransfected with an ERα expression plasmid, however, a strong ligand dependent response was seen with MD-MB-231, which had a similar drug response as MCF-7 cells. Surprisingly, transfected SKBR3 cells showed a reversed profile from that seen in MCF-7 cells. Raloxifene strongly stimulated SRF activity in SKBR3 cells transfected with ERα and estradiol repressed SRF activity. This suggests a new, cell context dependent pathway by which compounds that normally repress transcription at ERE promoters can activate transcription at other promoters.

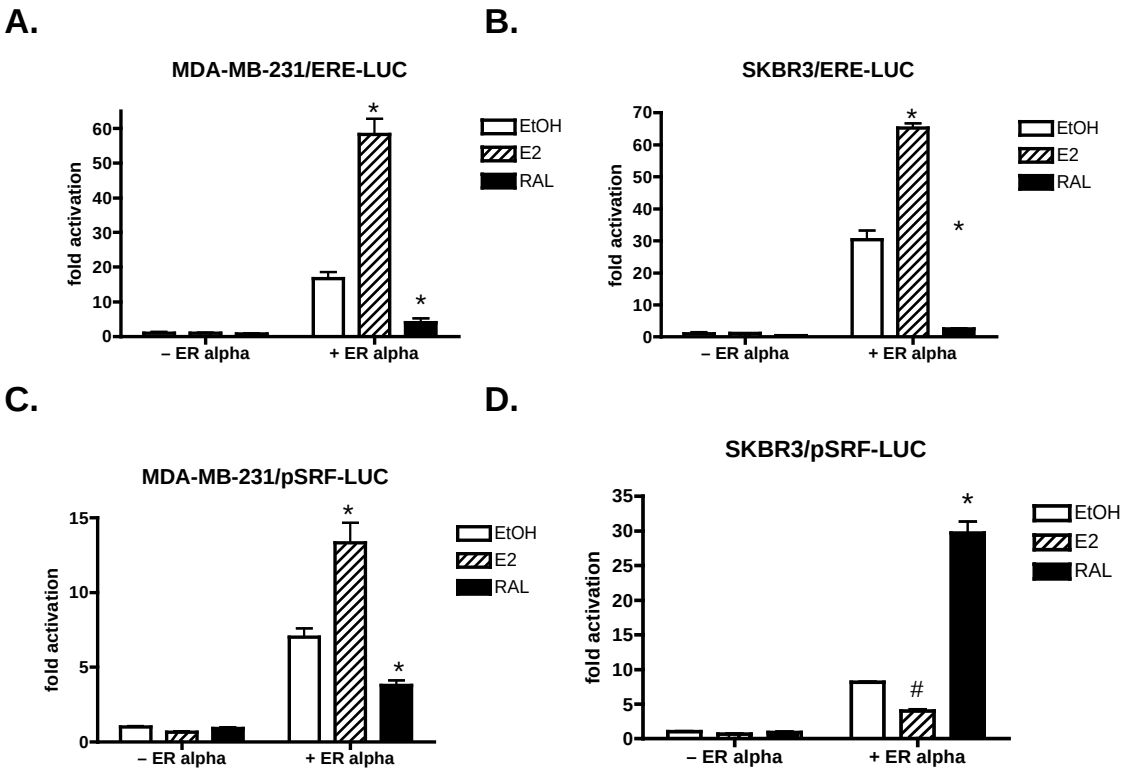



**Figure 8**. . Effect of ERα expression on MDA-MB-231 and SKBR3 cells transfected with a luciferase reporter plasmid containing either an SRF binding motif (A) or an ERE (B). All drugs were tested at 1 μM concentration. The cells were treated with drug for 24 hours in charcoal-stripped media. The results were obtained using a dual luciferase kit to normalize for cell number and transfection efficiency and the activity is reported as fold activation compared to the ethanol vehicle with no added ERα. * represents responses differing from the vehicle response with $p < 0.01$. # represents responses deviating from the vehicle response with $p < 0.05$.

Increasing concentrations of ERα expression vector increased the overall level of SRF-mediated activity, but the same relative level of stimulation by raloxifene compared to the vehicle control was observed, so the inversion of the raloxifene response in ERα-transfected SKBR3 cells compared to MCF-7 cells is not due to major differences in receptor expression. A normal dose response profile with raloxifene in these transfected SKBR3 cells was obtained with an EC50 equal to 2.2 ± 0.7 nM. This value is consistent with the binding affinity of raloxifene for ERα and also strongly suggests that the inversion of the raloxifene response in the SKBR3 cell line is not simply a non-specific response to high concentrations of raloxifene. In SKBR3 cells cotransfected with the ERα expression vector and the SRF reporter plasmid, estradiol antagonized activation of SRF activity by 10 nM raloxifene with an IC50 value equal to 2.4 ± 0.6 nM.

In order to better understand the signal transduction pathways by which ERα could be mediating its effects on SRF activity, inhibitors of different kinase signaling pathways were used. To determine whether PI3K or MAPK pathways played a role in the effects of ERα on SRF activity in our system, SKBR3 cells cotransfected with ERα and either the ERE or SRF reporter plasmid were

pretreated with either the PI3K inhibitor LY294002 or the MAP/ERK kinase (MEK) inhibitor U0126 and then treated with either raloxifene or estradiol. At the ERE-containing promoter, some reduction in the overall level of activation was seen for all drugs in the presence of either or both inhibitors, but the relative levels of activation for estradiol and raloxifene compared to vehicle was unchanged except for an increase in estradiol activation in the presence of the PI3K inhibitor (**Figure 9A**). In contrast, the overall transcriptional activity at the SRF reporter plasmid did not change significantly with inhibitor treatment, but the extent of raloxifene activation of SRF activity decreased approximately 30-40% in the presence of either the PI3K inhibitor or the MAPK inhibitor (**Figure 9B**). This strongly suggests that both the MAPK and PI3K pathways play a role in the stimulation of SRF activity by raloxifene and ERα. Unfortunately, addition of both inhibitors simultaneous was toxic to the cells, so the redundancy of the signaling pathways could not be explored. Interestingly, the repression of SRF activity by estradiol in ERα-transfected SKBR3 cells did not appear to be affected by either or both inhibitors, suggesting a different mechanism of action for estradiol repression compared to raloxifene activation.

**A.** **B.**

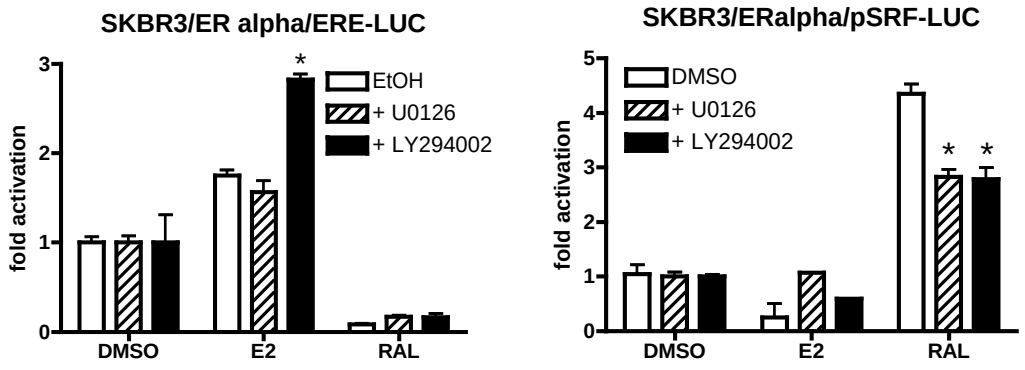



**Figure 9.** Effect of the MEK inhibitor U0126 or PI3K inhibitor LY294002 on expression of a luciferase reporter plasmid containing either an ERE (A) or SRF binding motifs (B) in ERα-transfected SKBR3 cells. All drugs were tested at 1 μM concentration. The cells were treated with drug for 24 hours in charcoal-stripped media. The results were obtained using a dual luciferase kit to normalize for cell number and transfection efficiency and results are reported as activation compared to the ethanol vehicle for each inhibitor * represents responses differing from the drug response with no kinase inhibitor added with $p < 0.01$.

In order to determine if raloxifene stimulation of SRF activity in ERα-transfected SKBR3 cells was a general response common to many SERMs, other compounds were tested. As described above (**Figure 3**), the SERM 4-hydroxytamoxifen and the SERDs fulvestrant and GW7604 had no effect on SRF activity in MCF-7 cells. In ERα-transfected SKBR3 cells, both fulvestrant and 4-hydroxytamoxifen showed stimulation of SRF activity, although not to the levels seen with raloxifene (**Figure 10**). In contrast, GW-7604 caused a significant repression of SRF activity in a manner similar to estradiol. This suggests that the transcriptional activity of SRF can be positively or negatively regulated by a variety of classes of ER-modulating compounds in a cell-context dependent manner.

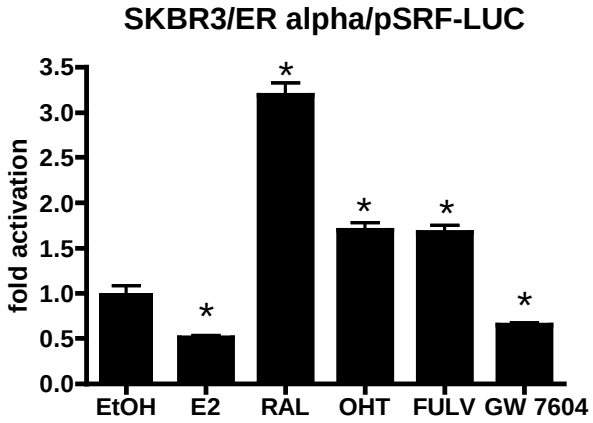



**Figure 10.** Effects of the ER ligand panel on SKBR3 cells transfected with an ERα expression plasmid and a luciferase reporter plasmid containing SRF binding motifs. All drugs were tested at 1 μM concentration. The cells were treated with drug for 24 hours in charcoal-stripped media. The results were obtained using a dual luciferase kit to normalize for cell number and transfection efficiency Activity is reported as fold activation compared to the ethanol vehicle. * represents responses differing from the vehicle response with $p < 0.01$.

There are still obviously a number of issues we must explore before claiming SRF reporter plasmid activity as a valid downstream assay for rapid estrogen signaling. The most important experiment is to correlate compound's ability to modulate ERK and Akt phosphorylation with its SRF profile. In MCF-7 cells, no correlation has been found between a compound's ability to stimulate ERK phosphorylation and its SRF activity, but previous reports suggest that SRF is regulated by PI3K in MCF-7 cells and not by MAPK [21,22]. The key experiments will be the Akt phosphorylation assays, which are underway. The major obstacle to determining whether SRF response in SKBR3 cells is downstream of rapid signaling is poor transfection efficiency of the estrogen receptor. If transfection efficiency is low, large number of cells that are not responding at all will dilute the overall extent of ERK and Akt phosphorylation assays in cells successfully transfected with the estrogen receptor. To get a better response, we are currently searching for an ER positive breast cancer cell line that shows the same profile as SKBR3 cells. This will allow us to determine the correlation between SRF activity and MAPK and PI3K activation and also determine whether there are correlations between the SRF response to different compounds and cell properties such as proliferation, resistance to apoptosis and antiestrogen resistance. Those studies are currently underway.

*Task 2. Test the potential role of estrogen receptor alpha (ER$\alpha$) or ER$\beta$ in rapid signaling, by performing assays with various N-terminal deletion and chimera mutants of ER$\alpha$ and ER$\beta$. (Months 12-36)*

All of the necessary mutants are prepared and active in luciferase reporter gene assays. This task will begin after a more robust rapid signaling assay is in place.

*Task 3. Test the role of cell surface receptors in rapid estrogen signaling, by developing cell-impermeable, non-proteinaceous estradiol conjugates.*

*Task 3a. Determine the feasibility of using polymer-conjugated estrogen ligands as probes of ER function by conjugating active estradiol & tamoxifen analogs to polymers synthesized using atom transfer radical polymerization (ATRP) and testing for their ability to bind to ER in vitro (Months 6-12)*

*Synthesis and ER binding*

Polymer scaffolds derived with bioactive molecules have been used for quite some time, in large part due to the advantage of being able to easily manipulate the bulk properties of the polymer.[24,25] A poly (methacrylate) polymer was synthesized as an N-hydroxysuccinimide activated ester using atom transfer radical polymerization (ATRP). ATRP allows for the generation of polymers with very narrow molecular weight ranges and the activated ester allows for conjugation of a number of different compounds through simple amide coupling chemistry- estrogenic compounds, antiestrogens and reporter groups like aminofluorescein and biotin. Activated polymers were synthesized in two sizes, 10,000 and 50,000 weight average molecular weight with polydispersity index numbers 1.1 and 1.5 respectively, conjugated with 1% aminofluorescein and then the remaining activated esters were hydrolyzed either to the carboxylic acid (**Figure 11**). The first generation polymer involved coupling the smaller polymer with compound **18** (n=6, R=H) and incorporating the compound into about 20% of the side chains. All of these polymers were dialyzed extensively in water and were water soluble at all the concentrations necessary for testing in the assays. The OHT conjugated polymer bound to the ERα with an IC50 equal to 123 ± 34 nM and to ERβ with an IC50 equal to 190 ± 15 nM. These are very potent binding affinities for a conjugate and will be useful for the biological assays. The estrone analogs are currently being synthesized.

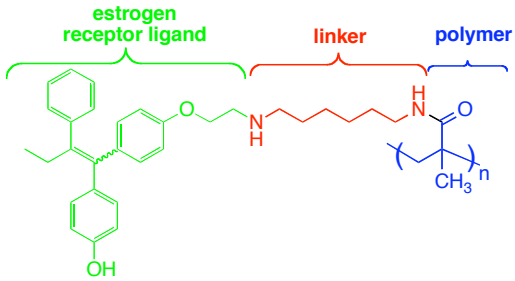



**Figure 11.** 4-hydroxytamoxifen conjugate used in initial studies

*Task 3b. Develop cell-impermeable polymer scaffolds suitable for cell-based assays by synthesizing well-defined polymers of different sizes and derivatizations from a single monomer unit using ATRP and testing for their general utility in biological screens. (Months 12-24)*

*Conjugate stability and protein binding*

The polymers were tested for their chemical and enzymatic stability. The fluorescein-containing polymer simplified these studies. Since these polymers

were larger than free fluorescein, a significant difference was seen in the fluorescence polarization values for the polymer-fluorescein conjugates compared to free fluorescein. Likewise, aggregation of the polymers by serum proteins should result in a much greater increase in the fluorescent polarization of the conjugates. Hydrolysis of the fluorescein from the polymers using concentrated NaOH, followed by neutralization resulted in samples with significantly lower polarization values. No change in fluorescence polarization was noted after the addition of 10% fetal bovine serum and incubation for 2 days at 37 ˚C. This suggests that the conjugates are relatively stable in serum and that large aggregates are not being formed between serum proteins and the conjugate.

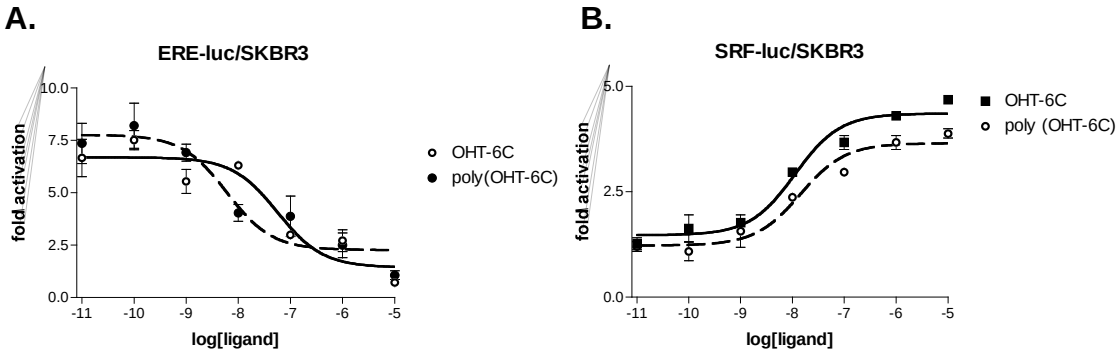



**Figure 12.** Activity of tamoxifen conjugates in cell-based assays. (A). Dose response curve of tamoxifen-polymer conjugate and unconjugated tamoxifen ligand vs. 10 nM estradiol in SKBR3 breast cancer cells transfected with ERα and luciferase reporter plasmid containing estrogen response element controlled promoter. (B). Dose response curve of tamoxifen-polymer conjugate and unconjugated tamoxifen ligand in SKBR3 breast cancer cells transfected with ERα and luciferase reporter plasmid containing serum response factor controlled promoter.

*Cell-impermeability*

The next key test for the conjugate was to determine whether they were cell impermeable as hypothesized. The first test of this permeability was an ERE reporter gene assay. If the conjugates were impermeable, no ERE signaling should be seen. Unfortunately, as shown in **Figure 12**, the conjugate was just as effective as the small molecule alone. This was also the case in SKBR3 cells transfected with the SRF reporter plasmid and ER$\alpha$. To determine whether cells were taking up the polymers, the fluorescent tamoxifen polymer conjugate was used. As shown in **Figure 13**, preliminary data show that the tamoxifen conjugate is present inside the cell in high amounts, suggesting that this scaffold is not cell impermeable as had been suggested by previous reports. Interestingly, cells take up the fluorescent tamoxifen conjugate to a greater degree than the non-tamoxifen conjugated polymer, suggesting the addition of tamoxifen actually increases uptake. Additionally, the addition of 4-hydroxytamoxifen greatly decreased the uptake of the polymer. This data are still preliminary, but they suggest that something is specifically transporting the conjugate into the cell. This would be an important (if somewhat serendipitous) discovery because tamoxifen was always considering to be taken up by cells via passive diffusion. An active transport process could potentially be studied further and manipulated

to potentially increase the efficacy of tamoxifen therapy. This issue will be explored further by looking at the extent of uptake in different breast cancer cell lines, the effect of the conjugate on cell growth and proliferation and determining whether uptake is altered in tamoxifen resistant breast cancer cell lines.

**A.** **B.**

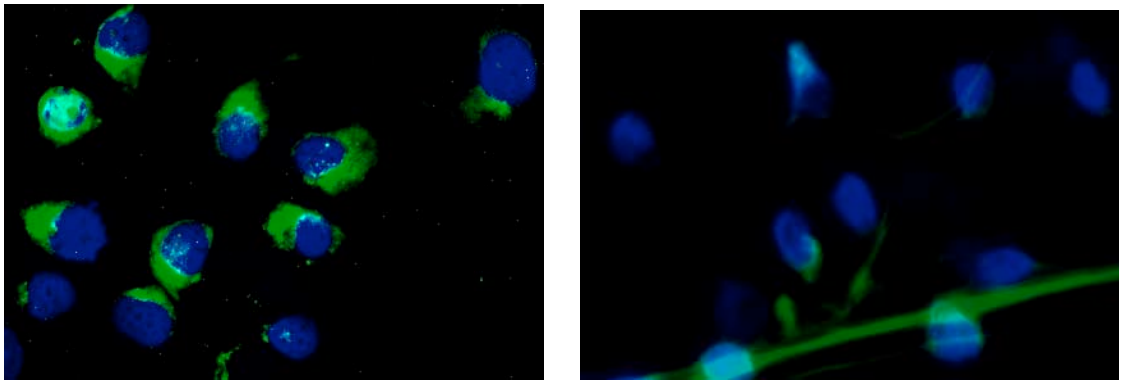


**Figure 13**. Uptake of fluorescein-tamoxifen-polymer conjugates. (A). MCF7 breast cancer cells treated with 50 nM fluorescein-tamoxifen-polymer conjugate for 1 hour, then fixed and stained with DAPI. Green represents polymer, blue represents DAPI-stained nuclei. (B). MCF7 breast cancer cells treated with 50 nM fluorescein-tamoxifen-polymer conjugate for 1 hour in the presence of 50 nM tamoxifen, then fixed and stained with DAPI. Green represents polymer, blue represents DAPI-stained nuclei

*Task 3c. After establishing the ideal polymer scaffold, active compounds will be coupled to the polymers and tested for their ability to elicit rapid steroid hormone responses in the different assays. (Months 24-36)*

The tamoxifen polymer conjugate was active in the ERK activation assay, but the current finding that the conjugates are not cell-impermeable has put further study on this question on hold until a better, more cell-impermeable conjugate is found (**Figure 14**). Current efforts are focused on using fluorescent nanocrystals (commonly referred to as quantum dots) coated with a polymer coating with free carboxylate groups [26]. Some nanocrystals have been used to target membrane receptors while other nanocrystals are taken into cells. Coupling of the tamoxifen conjugate went well and these particles are brightly fluorescent. Work is currently underway at determining their uptake and biological activity. Another approach that has been taken is to conjugate the tamoxifen analog to the highly polar Alexa-Fluor 486 Dye. This dye was conjugated to estradiol in order to localize GPR30 and was only able to bind to receptors inside the cell after cell permeabilization [11]. Synthesis of this compound is currently underway.

**A.** **B.**

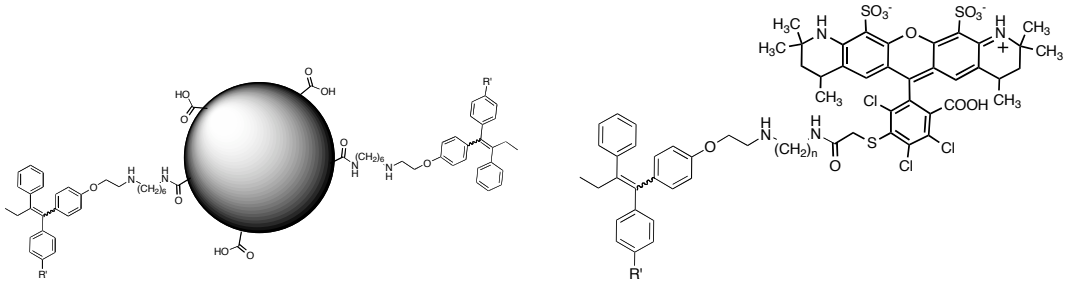


**Figure 14.** New attempts to design cell-impermeable ligands. (A.) schematic of polymer coated nanocrystals conjugates with 4-hydroxytamoxifen ligands. (B). Alexa 486-OHT conjugate.

*<u>Task 4</u>. Test the potential role of rapid estrogen signaling in breast cancer proliferation and survival, by treating various breast cell lines with selective compounds discovered above and measuring changes in cell growth, cytotoxicity and apoptosis (Months 24-36)*

Assays are in development, but no progress has been made on this task.

**Key Research Accomplishments (this year)**

- First reported example of specific uptake of tamoxifen conjugates by breast cancer cells.
- First example of conjugate targeting nuclear receptors that behave as highly potent transcriptional antagonists.
- New synthesis of GW-7604 and analogs that is much higher yielding than previously reported synthesis
- First reported discovery of cell-context dependent modulation of SRF transcriptional activity by estrogen receptor.
- First report of SERMs acting as strong activators of SRF signaling as well as first example of a unique response for GW-7604 compared to raloxifene and tamoxifen. .
- First synthesis of nanocrystals conjugated to steroid hormone receptor modulators.

**Reportable Outcomes (this year)**

*Manuscripts/abstracts (included in appendix)*

1. Trebley, J. P.; Rickert, E. L.; Reyes, P. T.; Weatherman, R. V., Tamoxifen-based Probes for the Study of Estrogen Receptor-Mediated Transcription. In *Chemical Genomics: Small Molecule Probes to Study Cellular Function*, Jaroch, S.; Hilmar, W., Eds. Springer: Berlin, 2006; Vol. 58, pp 76-87.

2. Weatherman, R. V., Untangling the estrogen receptor web. *Nat Chem Biol* **2006,** 2, 175-6.

*Presentations*

1. Speaker, American Association of Colleges of Pharmacy meeting, Cincinnati, July 2005.

2. Speaker, Department of Basic Medical Sciences, Indiana University-Bloomington, April 2005.

3. Speaker, Schering Foundation Workshop on Chemical Genomics, Berlin, Germany, April 2005.

4. Poster, Era of Hope Breast Cancer Research Meeting, Philadelphia, June 2005.

5. Poster, Purdue University Cancer Center Research Meeting, West Lafayette, August 2005.

6. Poster, American Society of Biochemistry and Molecular Biology Research Meeting, San Francisco, March 2006.

7. Poster, Midwest Endocrinology Conference Research Meeting, Madison, WI, June 2005.

8. Poster, Midwest Area Medicinal Chemistry graduate Student Symposium, Pittsburg, PA, June 2005.

*Patents and licenses applied for*

"Novel Triphenylethylethylene Analogs." Pre-disclosure form submitted to Purdue University Office of Technology Transfer

*Degrees obtained that are supported by this award*

Joseph Trebley, Ph.D. in Medicinal Chemistry and Molecular Pharmacology, 2006

*Funding applied for based on work supported by this award*

Received

none

Applied for

1. Kimmel Cancer Foundation (Principal Investigator)
2. National Institutes of Health, R01 (Principal Investigator)

*Employment or research opportunities applied for and/or received*

1. Joe Trebley, Ph.D., Technology Manager, Office of Technology Commercialization, Purdue University Research Foundation.
2. New collaborations with Yuliya Drobydneva, Eastern Virginia College of Medicine.

**Conclusions**

Currently, we have made significant progress in exploring the role of integrating nongenomic signaling in breast cancer prevention and treatment. We have synthesized all the planned compounds including a few more that were not in the original plan. We have had difficulty with finding robust and reproducible assays for the rapid response, but have discovered a number of new responses that might ultimately prove the importance of these responses. Most of the effort is currently focused on finding robust assays to measure these rapid responses, exploring the possible utility of our cell-permeable conjugates and designing new cell-impermeable conjugates. It is anticipated that the original plan will be completed within the next year.

In terms of the new knowledge we have obtained thus far and its importance to breast cancer, we have shown that SERMs like tamoxifen and raloxifene can act similarly to estrogen in activating rapid responses. We also have found a new cell context dependent response to estrogens and antiestrogens. This agonist activity of antiestrogens and the antagonist effects of estrogens mimic the effects of these drugs seen in some tamoxifen resistant tumors. In addition, we also found one compound (GW-7604) that is an antagonist in both cell contexts, suggesting that this compound could potentially be used to treat some tamoxifen resistant tumors. Understanding the molecular determinants of this agonist activity could help produce better treatments and chemopreventive agents for breast cancer.

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# 6 Tamoxifen-Based Probes for the Study of Estrogen Receptor-Mediated Transcription

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**Abstract.** The nuclear receptors are ideal targets to control the expression of specific genes with small molecules. Estrogen receptor can activate or repress transcription though a number of different pathways. As part of an effort to develop reagents that selectively target specific transcriptional regulatory pathways, analogs of 4-hydroxytamoxifen were synthesized with variations in the basic side chain. In vitro binding assays and cell-based luciferase reporter gene

assays confirm that all the derivatives have high affinity for the receptor and high potency at repressing direct estrogen receptor-mediated transcription.

## 6.1 Introduction

One of the ultimate goals of chemical genomics is to study the role of a specific protein by directly altering its activity with a small molecule. This could be performed either at the protein level by direct binding or at the transcriptional level by modulating the expression of its gene. Reagents such as small interfering RNA (siRNA) that block the production of protein have great utility, but small molecules that could either block or activate transcription of specific genes at specific time points would have a dramatic impact on discerning the role of a specific protein in cellular processes (Wang et al. 2004). One necessary component for developing these tools is a better understanding of the molecular mechanisms of transcriptional regulation and how small molecules can affect this complex process (Weatherman 2003).

Nuclear receptors such as the estrogen receptor (ER) represent an ideal system in which to study the effect of small molecules on the modulation of gene expression. Most nuclear receptors are ligand-dependent modulators of transcription, thus providing a tool to study the molecular mechanisms by which gene transcription is regulated. Nuclear receptors can activate or repress transcription upon ligand binding depending on the structure of the ligand, the nature of the promoter and the cell type (Katzenellenbogen et al. 1996). The estrogen receptor is a particularly interesting member of the nuclear receptor family because its effects on transcription can vary greatly depending on the ligand structure and the cellular context. For example, estradiol (**1**) has been shown to activate the expression of the *c-Myc* gene in breast cell lines and the breast cancer drug tamoxifen (**2**) antagonizes this activation (Shang and Brown 2002) (Fig. 1). In a uterine cell line, however, tamoxifen and estradiol both activate *c-Myc* expression. Other ER ligands with very similar structures to tamoxifen antagonize *c-Myc* expression in both types of cell lines. This tissue-dependent response profile of tamoxifen has therapeutic importance because the ER-agonist effects of tamoxifen in the uterus and in tamoxifen-resistant

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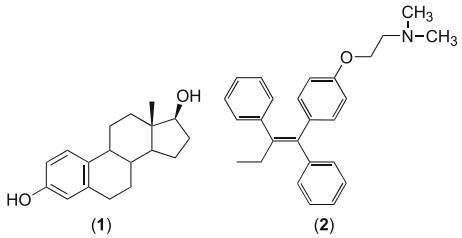



**Fig. 1.** Estradiol (**1**) and tamoxifen (**2**)

breast tumors are major obstacles to improving the success of tamoxifen therapy. These different response profiles allow for comparison of the different transcriptional states to help elucidate the molecular mechanisms underpinning the selective modulation of specific subsets of genes.

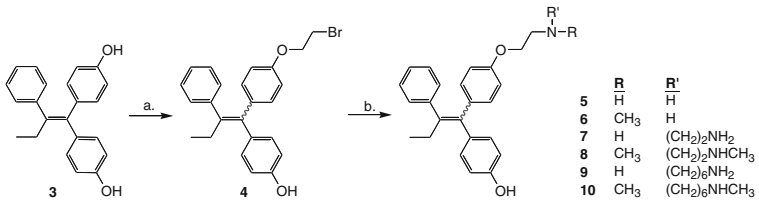
It is well known that estrogen receptor regulates gene transcription by binding to specific DNA sequences in the promoter region, but ER can also regulate gene transcription through indirect means. Estrogen receptor can directly interact with other transcription factors such as AP-1 and alter their activity, but it can also rapidly activate signal transduction proteins such as ERK and Akt, which can then activate downstream transcription factors such as Elk-1 and serum response factor (SRF) (Chen et al. 2004; Cheung et al. 2005). The activation of some of this rapid signaling occurs more prominently in cells in which tamoxifen acts as an estrogen receptor agonist, suggesting that the overall response profile of tamoxifen is tied to its ability to stimulate estrogen receptor crosstalk with other signal transduction pathways (Shah and Rowan 2005). Some evidence suggests that these rapid signaling events are initiated from the plasma membrane (Losel et al. 2003). Molecules that could selectively target only these crosstalk pathways would be very useful in delineating their role in the overall responses to tamoxifen. The work detailed here describes the synthesis and testing of tamoxifen analogs suitable for conjugation to other molecules such as fluorophores, affinity tags, and cell-impermeable polymer scaffolds in order to better understand the role of crosstalk signaling in the control of estrogen receptor-mediated transcription.

## 6.2 Results and Discussion

### 6.2.1 Synthesis of 4-Hydroxytamoxifen Analogs

The key issue in making tamoxifen analogs suitable for conjugation to other moieties is the placement of the attachment point. One obvious location for attachment is the amine on the basic side chain. Based on the structure of 4-hydroxytamoxifen, the most potent form of tamoxifen, bound to the ligand-binding domain of estrogen receptor alpha (ERα), the basic side chain extends out away from the interior of the binding pocket (Shiau et al. 1998). It has also previously been shown that endoxifen (**5**), a primary, bioactive metabolite of tamoxifen, can bind to the estrogen receptor both in vitro and in cells, with only small decreases in affinity compared to 4-hydroxytamoxifen (Johnson et al. 2004). Based on this evidence, a number of analogs of 4-hydroxytamoxifen with different lengths of alkylamine side chains were synthesized (Fig. 2).

The compounds were synthesized by using a modification of a previously reported synthesis of 4-hydroxytamoxifen (Yu and Forman 2003). The triphenylethylethylene scaffold can be synthesized as the diphenol (**3**) in a single step from commercially available starting materials and then monoalkylated with dibromoethane. The resulting compound (and every compound hereafter) is generated as a mixture of *E* and *Z* isomers, but the two forms readily interconvert at room temperature. Previous work with 4-hydroxytamoxifen has shown that despite this interconversion, the *Z* isomer is almost exclusively bound by the receptor both in vitro and in vivo (Katzenellenbogen et al. 1985).




| | R | R' |
|---|---|---|
| **5** | H | H |
| **6** | $CH_3$ | H |
| **7** | H | $(CH_2)_2NH_2$ |
| **8** | $CH_3$ | $(CH_2)_2NHCH_3$ |
| **9** | H | $(CH_2)_6NH_2$ |
| **10** | $CH_3$ | $(CH_2)_6NHCH_3$ |

**Fig. 2. a** $Cs_2CO_3$, DMF, 60 °C; 1,2 dibromoethane, 16 h. **b** RNHR′, THF, 60 °C, sealed tube, 12 h

Coupling to different amines provided the different compounds for testing. Since the optimal distance between the tamoxifen scaffold and any conjugate is not known, alkyldiamines with two and six methylene unit spacers were synthesized. Previous work has indicated that the methylation state of the amines could also be important in increasing the affinity of ligands for the estrogen receptor, so analogs with methylated amines were also synthesized.

### 6.2.2 In Vitro Binding Assays

The binding affinity of the compounds for estrogen receptor alpha was measured using a fluorescence polarization-based competition assay using purified full-length human estrogen receptor alpha. Displacement of a fluorescent ER ligand from the receptor by the competitor results in a decrease in the fluorescence polarization of the fluorophore. As shown in Fig. 3 and summarized in Table 1, all of the analogs had submicromolar affinities for the receptor. The only two compounds showing

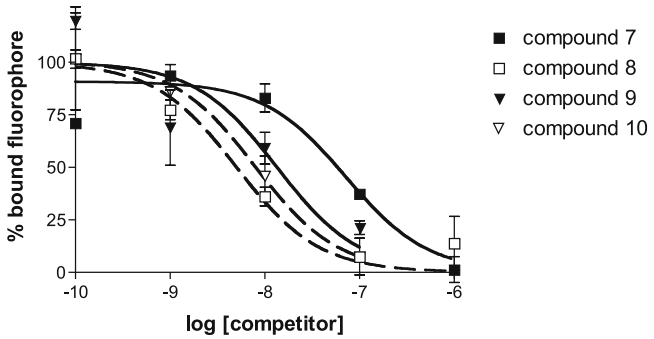



**Fig. 3.** Relative ER binding affinity of tamoxifen analogs **7–10**. The ability of various concentrations of different compounds to displace a synthetic fluorescent estrogen from recombinant preparations of ERα was evaluated as described in the material and methods section. *100* represents no displacement of fluorescent ligand, *0* represents total displacement. Each point represents the mean and standard error of the mean of three different samples. The *lines* represent the best fit to a single binding-site competition model. *Dashed lines* represent the fit for the methylated compounds

significantly different affinity for the receptor were the compounds with short extensions from the side chain terminating in primary amines (**5** and **7**). This could perhaps be due to some somewhat unfavorable interaction between the polar amine group and some nonpolar residues at the outer boundary of the binding pocket. A comparison of compounds **5** and **7** to compound **9** seems to indicate that pushing the primary amine further out of the binding pocket appears to be sufficient to overcome this unfavorable interaction.

### 6.2.3 Cell-Based Reporter Assays

The ability of the compounds to modulate estrogen receptor-mediated gene transcription was tested using a luciferase reporter gene assay. The ER-negative HeLa cervical cell line was transiently transfected with a plasmid expressing human ERα and a plasmid containing the luciferase gene under the control of the vitellogenin promoter. This promoter contains two consensus estrogen receptor binding sites and is activated strongly in the presence of ER and estradiol. None of the compounds showed any agonist activity (data not shown), so antagonist activity was determined by performing competition assays in the presence of 10 nM estradiol. As shown in Fig. 4 and summarized in Table 1, the compounds were all antagonists of estradiol-induced ER activation at the vitellogenin promoter at relatively low concentrations. Although the variability between assays is much greater with cell-based

**Table 1.** Summary of $K_i$ values for compounds calculated from the receptor competition experiments and $IC_{50}$ values vs 10 nM estradiol calculated from the reporter gene assays

| Compound | $K_i$ (nM) | $IC_{50}$ (nM) |
|---|---|---|
| Estradiol (**1**) | $6.3 \pm 0.2$ | N.D. |
| **5** | $48 \pm 5$ | $800 \pm 400$ |
| **6** | $8.5 \pm 3.9$ | $40 \pm 10$ |
| **7** | $32 \pm 10$ | $150 \pm 50$ |
| **8** | $3.4 \pm 2.1$ | $39 \pm 12$ |
| **9** | $9.8 \pm 6.2$ | $85 \pm 55$ |
| **10** | $6.2 \pm 4.6$ | $126 \pm 33$ |

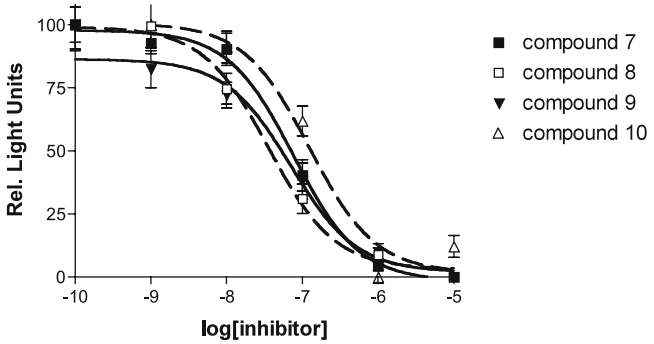



**Fig. 4.** Competition of the compounds **7–10** vs 10 nM estradiol in transient transfection assay of HeLa cells with ERα and the vitellogenin A2 ERE-tk-driven luciferase reporter gene. The *curve* represents the best fit to a single-site competition binding model. 100% activation represents the activation with 10 nM estradiol alone. Each point represents the mean and standard error of the mean of three different samples. *Solid lines* represent the best fit to a single binding-site competition model. *Dashed lines* represent the fit for the methylated compounds

assays than with the in vitro binding assay, compound **5** showed a significant decrease in antagonist potency compared to the other compounds. Whether this decrease is due to weaker binding affinity for the receptor or diminished cell uptake is unknown. Overall, however, all of the tamoxifen analogs inhibited ER-mediated transcriptions at concentrations that are low enough to allow for future derivatization studies.

### 6.2.4 Conclusion

In summary, a novel set of tamoxifen analogs has been made using a relatively simple synthetic scheme. Receptor affinity assays and reporter gene assays indicate that many of the analogs have potencies similar to tamoxifen and would make suitable analogs to conjugate to other moieties in order to study roles of the different pathways leading to estrogen receptor-mediated transcriptional regulation. These moieties will include fluorescent molecules that will allow for the visualization of binding either inside the cell or on the cell surface. The analogs

will also be conjugated to cell-impermeable polyacrylate polymers that should allow for selective targeting of membrane-initiated responses of estrogen receptor. It is envisioned that these tools will help elucidate the pleiotropic behavior of tamoxifen and could be used in the future to help engineer novel transcription factors that could either activate or repress the transcription of specific genes.

## 6.3 Materials and Methods

### 6.3.1 General Methods

All reagents were purchased from Sigma-Aldrich. The expression plasmids used in this study, pSG5-ERα and ERE-luciferase, were generously provided by Thomas Scanlan (UCSF) and have been described elsewhere (Weatherman et al. 2001; Weatherman and Scanlan 2001). The ERE-driven luciferase reporter gene consists of two repeats of the upstream region of the vitellogenin ERE promoter from −331 to −289, followed by region −109 to +45 of the thymilidate kinase upstream region and the luciferase gene. Proton and $^{13}$C nuclear magnetic resonance spectra (1H NMR, 13C NMR) were obtained on a Bruker ARX300 (300 MHz) instrument; 1H NMR chemical shifts are reported as δ values in parts per million (ppm) downfield from internal tetramethylsilane. The 13C NMR chemical shifts are reported as δ values with reference to the solvent peak. Mass spectrometry (MS) and NMR instruments were provided by the Shared Resource center of the Purdue Cancer Center.

### 6.3.2 Synthesis of Tamoxifen Analogs

***E* and *Z***
**4-{1-[4-(2-Bromo-ethoxy)-phenyl]-2-phenyl-but-1-enyl}-phenol (4)**

Diphenol (**3**) (0.5 g, 1.59 mmol) (Yu and Forman 2003) was dissolved in DMF (10 ml) and then cesium carbonate (2.07 g, 6.4 mmol, 4 equiv.) was added and the solution was heated at 60 °C for 15 min. The 1,2 dibromoethane (0.5 ml, 5.7 mmol, 4.5 equiv.) was then added all at once and the reaction was allowed to stir for 16 h at 60 °C. We then added 30 ml of water to the reaction mixture and the compounds were extracted

with ethyl acetate twice. The organic layer was washed with brine, dried with magnesium sulfate, and then the solvent was evaporated under reduced pressure. Purification by flash silica gel chromatography using 30% ethyl acetate in hexane as the eluent provided 0.25 g of desired product (0.59 mmol, 37% yield) as a mixture of interconverting *E* and *Z* isomers. 1H NMR (300 MHz) (CDCl3) δ 7.15 (7H, m) δ 6.94 (2H, d) δ 6.83 (1H, dd) δ 6.78 (1H, d) δ 6.62 (1H, d) δ 6.56 (1H, d) δ 4.69 (1H, t) δ 4.57 (1H, t) δ 4.12 (1H, t) δ 4.01 (1H, t) δ 3.10 (2H, q) δ 1.77 (3H, t); 13C NMR (300 MHz) (CDCl3) δ 157.18, δ 153.83, δ 142.95, δ 141.704, δ 138.01, δ 137.48, δ 136.29, δ 132.55, δ 131.15, δ 130.13, δ 128.24, δ 126.42, δ 115.43, δ 114.75, δ 114.00, δ 68.30, δ 29.68, δ 14.06. MS (CI) m/z 423/425 $(M + H)^+$.

**General Synthesis of Amine Analogs**

The bromide (**5**) (50 mg, 0.12 mmol) was dissolved in THF (2 ml) and 0.5 g of the appropriate diamine (as described below) was then added and the solution was heated at 60 °C for 12 h in a sealed tube. The solvent was then removed, evaporated under reduced pressure, and then purified by silica gel flash chromatography using 5.5/4/0.5 $CHCl_3/CH_3OH/NH_4OH$ as the eluent provided the product as a mixture of interconverting *E* and *Z* isomers. Below is information for each compound:

***E* and *Z***
**4-{1-[4-(2-Aminoethoxy)-phenyl]-2-phenyl-but-1-enyl}-phenol (5)**

$NH_4OH$ was used as the amine and 43 mg of purified product was isolated (0.11 mmol, 92% yield). 1H NMR (300 MHz) (CDCl3) δ 7.15 (7H, m) δ 6.88 (1H, d) δ 6.81 (2H, dd) δ 6.72 (1H, d) δ 6.58 (1H, d) δ 6.52 (1H, d) δ 4.51 (1H, t) δ 4.37 (1H, t) δ 3.58 (1H, t) δ 3.49 (1H, t) δ 3.12 (5H, m) δ 2.02 (1H, s) δ 1.76 (3H, t). MS (CI) m/z 360 (M + H).

***E* and *Z* 4-{1-[4-(2-Methylaminoethoxy)-phenyl]-2-phenyl-but-1-enyl}-phenol (6)**

2 M methylamine in THF was used as the amine and 35 mg of purified product was isolated (0.094 mmol, 78% yield). 1H NMR (300 MHz) (CDCl3) δ 7.15 (7H, m) δ 6.88 (1H, d) δ 6.81 (2H, dd) δ 6.72 (1H, d) δ 6.58 (1H, d) δ 6.52 (1H, d) δ 5.76 (2H, s) δ 4.51 (1H, t) δ 4.37 (1H, t)

δ 3.58 (1H, t) δ 3.49 (1H, t) δ 3.12 (5H, m) δ 2.02 (1H, s) δ 1.76 (3H, t); 13C NMR (300 MHz) (CDCl3) δ 157.61, δ 156.77, δ 156.26, δ 155.34, δ 143.18, δ 141.20, δ 138.43, δ 137.28, δ 136.78, δ 135.47, δ 135.10, δ 132.43, δ 131.13, δ 130.16, δ 128.26, δ 126.27, δ 115.71, δ 115.04, δ 114.40, δ 113.65, δ 66.56, δ 50.81, δ 36.14, δ 29.50, δ 14.11. MS (CI) m/z 374 (M + H).

**_E_ and _Z_ 4-(1-{4-[2-(2-Aminoethylamino)-ethoxy]-phenyl}-2-phenyl-but-1-enyl)-phenol (7)**

Ethylenediamine was used as the amine and 32 mg of purified product was isolated (0.087 mmol, 73% yield). 1H NMR (300 MHz) ($CD_3OD$) δ 7.15 (7H, m) δ 6.88 (1H, d) δ 6.81 (2H, dd) δ 6.72 (1H, d) δ 6.58 (1H, d) δ 6.52 (1H, d) δ 4.51 (1H, t) δ 4.37 (1H, t) δ 3.58 (3H, t) δ 3.49 (3H, t) δ 3.12 (5H, m) δ 2.02 (1H, s) δ 1.76 (3H, t); 13C NMR (300 MHz) ($CD_3OD$) δ 159.4, δ 158.5, δ 157.9, δ 157.0, δ 144.6, δ 142.4, δ 142.2, δ 140.2, δ 138.43, δ 137.6, δ 136.3, δ 133.47, δ 132.43, δ 131.13, δ 130.16, δ 128.26, δ 126.27, δ 115.71, δ 115.04, δ 114.40, δ 113.65, δ 66.56, δ 42.13, δ 31.2, δ 29.50, δ 14.11. MS (CI) m/z 403 (M + H).

**_E_ and _Z_ 4-[1-(4-{2-[Methyl-(2-methylaminoethyl)-amino]-ethoxy}-phenyl)-2-phenyl-but-1-enyl]-phenol (8)**

N,N′ dimethylethylenediamine was used as the amine and 15 mg of purified product was isolated (0.035 mmol, 29% yield). 1H NMR (300 MHz) ($CDCl_3$) δ 7.15 (7H, m) δ 6.88 (1H, d) δ 6.81 (2H, dd) δ 6.72 (1H, d) δ 6.58 (1H, d) δ 6.52 (1H, d) δ 4.37 (1H, t) δ 4.12 (3H, t) δ 3.95 (3H, t) δ 3.6 (5H, m) δ 2.58 (3H, s), δ 2.50 (3H, s), δ 2.02 (1H, s) δ 1.76 (3H, t).

**_E_ and _Z_ 4-(1-{4-[2-(6-Amino-hexylamino)ethoxy]-phenyl}-2-phenyl-but-1-enyl)-phenol (9)**

1,6-diaminohexane was used as the amine and 40 mg of purified product was isolated (0.092 mmol, 77% yield). 1H NMR (300 MHz) ($CDCl_3$) δ 7.15 (7H, m) δ 6.88 (1H, d) δ 6.81 (2H, dd) δ 6.72 (1H, d) δ 6.58 (1H, d) δ 6.52 (1H, d) δ 3.6 (5H, m) δ 2.58 (2H, t), δ 2.50 (2H, t), δ 2.02 (1H, s) δ 1.6 (3H, t), δ 1.3 (8H, m).

***E* and *Z* 4-[1-(4-{2-[Methyl-(6-methylaminohexyl)-amino]-ethoxy}-phenyl)-2-phenyl-but-1-enyl]-phenol (10)**

N,N′ dimethyl-1,6-diaminohexane was used as the amine and 18 mg of purified product was isolated (0.037 mmol, 31% yield). 1H NMR (300 MHz) ($CDCl_3$) δ 7.15 (7H, m) δ 6.88 (1H, d) δ 6.81 (2H, dd) δ 6.72 (1H, d) δ 6.58 (1H, d) δ 6.52 (1H, d) δ 3.2 (2H, t) δ 3.1 (2H, t), δ 2.55 (2H, t), δ 2.45 (6H, s), δ 2.22 (2H, t) δ 1.6 (3H, m), δ 1.3 (8H, m).

### 6.3.3 Fluorescence Polarization Assay

Fluorescent polarization-based competition binding assays were conducted to determine the relative affinity of the 4-hydroxytamoxifen analogs for ERα using a commercially available kit (PanVera Corp., Madison, WI). Briefly, serial dilutions of the different compounds were prepared in ES2 screening buffer (100 mM potassium phosphate, pH7.4, 100 μg/ml bovine gamma globulin) and 50 μl of each concentration was aliquoted into three wells of a black 96-well assay plate. Fifty microliters of a solution containing 20 nM recombinant ERα, and 2 nM of a proprietary fluorescent ER ligand (Fluormone-ES2) was added to each well. The plate was shaken on a plate mixer and incubated for 2 h in the dark at room temperature. Fluorescence polarization signals were then measured using a Packard Fusion fluorimeter. The data were then fit to a single binding site competition curve by nonlinear regression analysis (Prism 3 software package). $K_i$ values were determined from the average of three different experiments and calculated using a $K_D = 4$ nM for Fluormone binding to ERα.

### 6.3.4 Cell Culture and Transient Transfection Experiments

#### Cell Culture

HeLa cells were obtained from the American Type Culture Collection (ATCC). HeLa cells were maintained in DME media without phenol red (Sigma) supplemented with 4.5 g/l glucose, 0.876 g/l glutamine, 100 mg/l streptomycin sulfate, 100 units/ml of penicillin G, and 10% FBS at 37 °C in an air/carbon dioxide (95:5) atmosphere. Transfection

assays were run with the same media conditions except the FBS was treated for 24 h with dextran-coated charcoal.

### Transient Transfection Assays

HeLa cells were plated in 24-well plates and grown to approximately 70%–80% confluency. Transfections were performed according to the protocol for Lipofectamine 2000 (Invitrogen). In order to normalize for the transfection efficiency in each well, the dual luciferase system was used in which a constitutively expressed, chemically orthogonal luciferase expression vector was also transfected. The total amount of DNA/well for each plasmid was as follows: pSG5-ERα 0.25 μg/well, ERE-luciferase 0.5 μg/well, and *Renilla*-luciferase 0.25 μg/well. The ratio of total DNA/Lipofectamine 2000 was 1:5. After transfection, the plates incubated at 37 °C for 6 h before dosing with drug. All drugs were delivered in DMSO or ethanol and the total concentration of organic solvent in each was 0.1%. For competition experiments, the drug was added to media already containing 10 nM estradiol. After 18–24 h, the cells were lysed and assayed for dual luciferase activity in a TopCount luminometer according to the protocol provided by Promega. The relative light units (RLU) were then calculated by dividing the output of the ERE-driven luciferase in each well by the output of the *Renilla* luciferase. Each drug concentration was tested in triplicate.

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# Untangling the estrogen receptor web

Ross V Weatherman

**GPR30, a G-protein coupled receptor, is a recent addition to the family of receptors that bind to estrogens and antiestrogens. A new, selective compound for GPR30 has been developed to dissect the role of GPR30 in estrogen signaling.**

Drugs that affect estrogen signaling pathways are taken by millions of women for contraception as well as the treatment of menopausal symptoms and breast cancer. Even with such widespread use, there are many fundamental questions about how these drugs work, including the nature of the receptors that mediate the drugs' effects. The newest member of the estrogen receptor family is GPR30, a G-protein coupled receptor (**Fig. 1**). In this issue of *Nature Chemical Biology*, Bologa *et al.*[1] report the discovery of a compound that selectively targets GPR30 over other estrogen receptors. This compound should be a valuable tool in elucidating the role of GPR30 in estrogen hormone signaling and possibly lead to better therapeutic agents.

A signature of drugs that target estrogen signaling is that the drugs can act as estrogen mimics in some tissues while acting as antiestrogens in others.[2] Tamoxifen is used for the treatment of breast cancer because it is an antiestrogen in breast tissue, but tamoxifen's therapeutic effectiveness is limited because it acts as an estrogen in uterine tissue, slightly increasing a woman's risk for uterine cancer. Attaining the ideal tissue response profile for a specific therapeutic indication is difficult because the mechanisms for these responses are poorly understood. Deciphering the pharmacology of estrogen receptors is key to the development of better agents.

It was believed that estrogen action was mediated exclusively through an intracellular, ligand-activated transcription factor that regulated the transcription of subsets of genes. In 1996, this receptor, now known as estrogen receptor alpha (ERα), was joined by another related receptor named estrogen receptor beta (ERβ) that has many similar functions as ERα as well as some unique functions of its own.[3] Compounds that selectively target ERβ elicit interesting anti-inflammatory effects in a number of chronic inflammation models, but thus far have not exhibited the therapeutic responses normally associated with traditional estrogen drugs.[4]

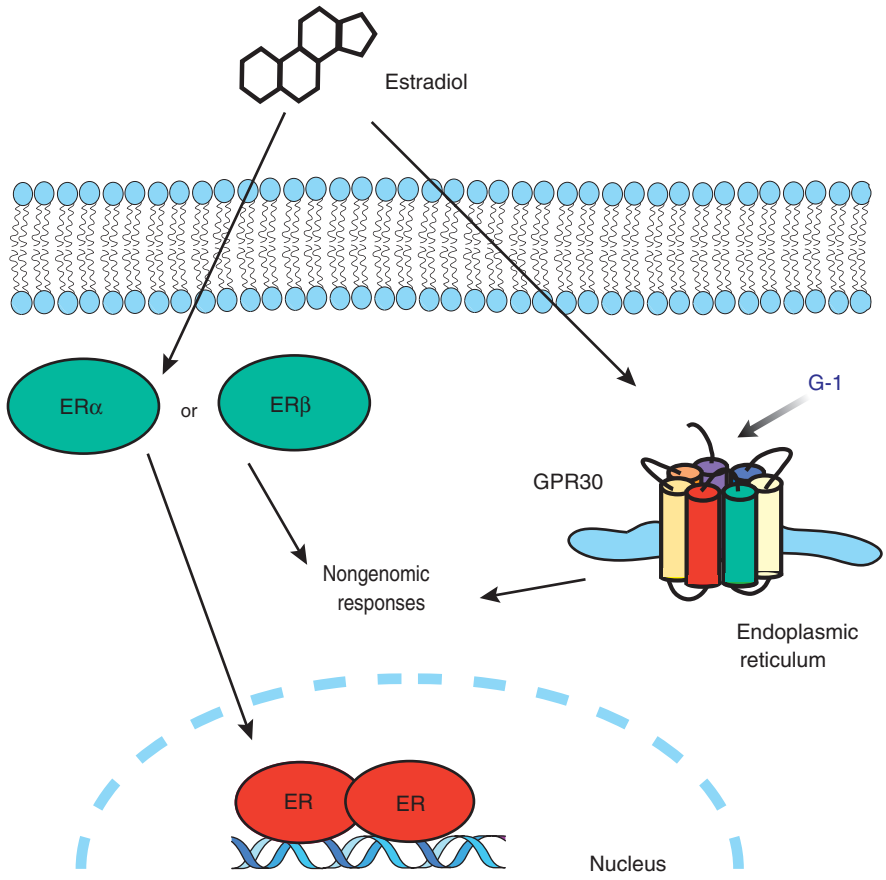


**Figure 1** Estrogen signaling pathways in a cell. Estradiol can bind to either estrogen receptor alpha or beta or GPR30. After ligand binding, the nuclear receptors can translocate to the nucleus and regulate transcription. Both the nuclear receptors and GPR30 are known to modulate nongenomic signaling pathways. The newly reported compound G1 binds selectively to GPR30.

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More recently, models of estrogen action have emerged that do not exclusively involve gene transcription and instead involve rapid activation of other signaling pathways such as kinase phosphorylation and intracellular calcium release.[5] These responses, sometimes referred to as nongenomic responses, are usually associated with other receptor classes such as receptor tyrosine kinases or G-protein coupled receptors. In this context, the expression of GPR30, a G-protein coupled receptor with no previously reported ligand, was linked to the activation of certain kinase signaling pathways by estradiol.[6] Concurrently, it was also shown that ERα and ERβ could also activate nongenomic signaling through crosstalk with other signal transduction proteins. The exploration of ERα and ERβ regulation of nongenomic signaling has largely overshadowed work on GPR30 until 2005, when it was revealed that GPR30 mediated estrogen responses while localized to the endoplasmic reticulum inside the cell.[7] This property is unique to GPR30 in all of the G-protein coupled receptors studied to date. This report generated some criticism since no biological role had ever been demonstrated for GPR30.[8] Unfortunately, the ubiquity of ERα and ERβ makes it almost impossible to study the role of GPR30 alone in normal cellular contexts. The solution lies in the development of a GPR30 selective compound, which Bologa and coworkers report in this issue.

The authors prescreened 10,000 molecules by computational methods for G-protein coupled receptor binding ability and then tested the 100 best molecules using an assay in which either ERα, ERβ or GPR30 was expressed in cells devoid of any estrogen receptors. The cells were then treated with a fluorescent conjugate of estradiol and the compound of interest and the binding of the fluorescent conjugate to the receptor in individual cells was measured using flow cytometry. One compound, which the authors named G-1, displaced the conjugate from cells expressing GPR30 but not from cells expressing ERα or ERβ. Further testing showed the compound exhibited greater than 1000-fold preference for GPR30 compared to the nuclear receptors and could stimulate intracellular calcium mobilization, activation of phosphoinositide kinase signaling and inhibition of cell migration in GPR30-expressing cells.

Armed with this compound, researchers should be able to learn a great deal more about the role of GPR30 in estrogen action. Ideally, a GPR30 selective antagonist and an estrogen receptor binding compound that excludes GPR30 can also be developed to provide more experimental flexibility to those interested in studying GPR30. Ultimately, it is almost certain that some, if not most, of the functions of GPR30 will crosstalk with the nongenomic and transcriptional activities of ERα and ERβ. Untangling this web of receptor interactions will ultimately lead to better understanding of estrogen function and possibly the development of more specific therapeutic agents with fewer side effects.


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